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# **ABCC1 confers tissue-specific sensitivity to cortisol versus corticosterone: a rationale for safer glucocorticoid replacement therapy**

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1           **Title: ABCC1 confers tissue-specific sensitivity to cortisol versus**  
2           **corticosterone: a rationale for safer glucocorticoid replacement therapy**

3  
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23

24 **One Sentence Summary:** Corticosterone is excluded from adipose tissue by the trans-  
25 membrane transporter ABCC1 and is as effective as cortisol for ACTH suppression, but lacks  
26 metabolic adverse effects.

27 **Abstract**

28 The aim of treatment in congenital adrenal hyperplasia is to suppress excess adrenal androgens  
29 while achieving physiological glucocorticoid replacement. However, current glucocorticoid  
30 replacement regimes are inadequate, because doses sufficient to suppress excess androgens  
31 almost invariably induce adverse metabolic effects. Although both cortisol and corticosterone are  
32 glucocorticoids that circulate in human plasma, any physiological role for corticosterone has  
33 been neglected. In the brain, the ATP-binding cassette transporter ABCB1 exports cortisol but  
34 not corticosterone. Conversely, ABCC1 exports corticosterone but not cortisol. We show that  
35 *ABCC1* but not *ABCB1* is expressed in human adipose, and that ABCC1 inhibition increases  
36 intracellular corticosterone but not cortisol and induces glucocorticoid-responsive gene  
37 transcription, in human adipocytes. Both C57Bl/6 mice treated with the ABCC1 inhibitor  
38 probenecid and FVB mice with deletion of *Abcc1* accumulated more corticosterone than cortisol  
39 in adipose after adrenalectomy and corticosteroid infusion. This accumulation was sufficient to  
40 increase glucocorticoid-responsive adipose transcript expression. In human adipose tissue, tissue  
41 corticosterone concentrations were consistently low, and *ABCC1* mRNA was upregulated in  
42 obesity. To test the hypothesis that corticosterone effectively suppresses ACTH without the  
43 metabolic adverse effects of cortisol, we infused cortisol or corticosterone in patients with  
44 Addison's disease. ACTH suppression was similar, but subcutaneous adipose transcripts of  
45 glucocorticoid-responsive genes was higher after cortisol than corticosterone. These data indicate  
46 that corticosterone may be a metabolically favorable alternative to cortisol for glucocorticoid

- 47 replacement therapy when ACTH suppression is desirable, as in congenital adrenal hyperplasia,
- 48 and justify development of a pharmaceutical preparation.

2012

49 **Introduction**

50 Congenital adrenal hyperplasia (CAH) is characterized by impaired adrenal steroidogenesis,  
51 with glucocorticoid deficiency resulting in reduced suppression of adrenocorticotrophic  
52 hormone (ACTH), and hence ACTH-dependent adrenal androgen excess. Current treatment  
53 guidelines recommend cortisol (hydrocortisone) as the treatment of choice, but recent studies  
54 have found that the doses required to suppress adrenal androgen production are usually  
55 associated with adverse effects (1, 2), notably in adipose tissue where chronic glucocorticoid  
56 excess promotes obesity and associated metabolic dysfunction (3). The ideal treatment would  
57 have higher potency in suppressing ACTH and lower potency as a glucocorticoid acting in  
58 peripheral tissues. Here, we provide a rationale and proof of concept for using corticosterone  
59 therapy as an alternative to hydrocortisone (cortisol).

60 A neglected characteristic of the hypothalamus-pituitary-adrenal (HPA) axis is that  
61 many species produce two glucocorticoids, cortisol and corticosterone, unlike rats and mice  
62 that lack 17-hydroxylase (CYP17) in their adrenals and therefore secrete only corticosterone.  
63 Although results of human glucocorticoid receptor binding studies are inconsistent (4, 5),  
64 both glucocorticoids appear to have similar affinities for mineralocorticoid receptors (6) and  
65 are subject to similar metabolism (7). However, there is emerging evidence for tissue-specific  
66 responses to cortisol and corticosterone, mediated by differential susceptibility to  
67 transmembrane transport by ATP-binding cassette (ABC) transporters (8-12). In rodents,  
68 ABCB1 (also known as multidrug resistance protein 1, MDR1, or p-glycoprotein) exports  
69 cortisol but not corticosterone across the blood-brain barrier (10, 12). Disproportionately high  
70 corticosterone concentrations in cerebrospinal fluid and brain tissue suggest that the same

71 mechanism operates in humans (10, 13). Another transporter, ABCC1 (or multidrug  
72 resistance-related protein 1, MRP1), exports corticosterone but not cortisol in vitro (11). We  
73 show expression of ABCC1 but not ABCB1 in human adipose tissue, report in vitro and in  
74 vivo data supporting the hypothesis that adipose tissue excludes corticosterone and is  
75 preferentially sensitive to cortisol, and provide proof of concept that corticosterone may be a  
76 useful therapy especially when suppression of ACTH is desirable, as in CAH.

77 **Results**

78 *ABCC1 but not ABCB1 is expressed in human adipose*

79 In a transcriptomic analysis of ABC transporters in healthy men, *ABCC1* but not *ABCB1* was  
80 highly expressed in subcutaneous adipose tissue (Figure 1A and figure S1). This pattern was  
81 confirmed by conventional reverse transcriptase polymerase chain reaction amplification in  
82 human adipose tissue and in differentiated SGBS human adipocytes (Figure 1B) (14), in which  
83 we also confirmed that *ABCC1* protein and mRNA are regulated in parallel, for example when  
84 we induced *ABCC1* by incubation with lipopolysaccharide (figure S2). However, in murine  
85 adipose tissue, both *ABCB1* and *ABCC1* were expressed (Figure 1C). Publicly available datasets  
86 do not provide sufficient data to test for patterns of *ABCB1* and *ABCC1* expression in multiple  
87 species, but we also found selective adipose expression of *ABCC1* but not *ABCB1* in horse,  
88 another species in which both cortisol and corticosterone are in the circulation (Figure 1D).

89 *ABCC1 preferentially exports corticosterone and confers sensitivity to cortisol in human*  
90 *adipocytes in vitro*

91 After incubation of SGBS adipocytes with cortisol or corticosterone for 24 hours, removal of  
92 extracellular steroid resulted in lower intracellular concentrations of corticosterone compared to  
93 those of cortisol over the subsequent 24 hours (Figure 2A), suggesting active preferential export  
94 of corticosterone. This was confirmed and attributed to *ABCC1* using the inhibitors MK571 (15)  
95 and probenecid (11). MK571 concentrations (10  $\mu$ M) sufficient to inhibit *ABCC1*-dependent  
96 export of calcein by ~40% in SGBS cells (figure S3A) (16) increased accumulation of



97 intracellular deuterated corticosterone assessed qualitatively using coherent anti-Stokes Raman  
98 scattering (CARS) microscopy (Figure 2B). Similarly, both MK571 and probenecid increased  
99 intracellular tritiated corticosterone much more so than tritiated cortisol (Figure 2C). Effects of  
100 MK571 and probenecid on corticosterone retention were time-dependent (figure S3B) and dose-  
101 dependent (figure S3C,D).

102 To test the consequences of ABCC1-mediated steroid transport for glucocorticoid receptor (GR)  
103 activation, we used A549 human epithelial cells (which express ABCC1 and GR) transfected  
104 with a glucocorticoid-responsive MMTV-luciferase reporter (17). The response was greater to  
105 cortisol than corticosterone without inhibition of ABCC1, and MK571 potentiated luciferase  
106 induction by corticosterone but not cortisol (figure S4A). In differentiated SGBS adipocytes, we  
107 tested a panel of known GR-responsive gene transcripts to determine a suitable candidate for  
108 assessing acute cortisol versus corticosterone sensitivity (Figure 2D). Of these, only *PER1* was  
109 induced acutely (2 hours) by glucocorticoids, consistent with its rapid response (18) and high  
110 sensitivity (19) in other systems. We showed greater sensitivity to cortisol than corticosterone for  
111 induction of *PER1* transcription (Figure 2D). MK571 directly increased the amount of this  
112 transcript (figure S4B), so probenecid was used alone as an ABCC1 inhibitor, reversing the  
113 differential sensitivity to cortisol over corticosterone (Figure 2D). Similar results were obtained  
114 after 24 hour incubations (figure S4C). Finally, to confirm whether adipocytes are functionally  
115 more sensitive to cortisol than corticosterone, SGBS preadipocytes were differentiated in the  
116 presence of glucocorticoid; cortisol but not corticosterone induced marked triglyceride  
117 accumulation over 21 days (Figure 2E).

118

119 *Genetic disruption or pharmacological inhibition of Abcc1 in mice results in preferential*  
120 *accumulation of corticosterone in adipose tissue*

121 Male control mice (*Abcc1*<sup>+/+</sup>) and mice deficient in *Abcc1* (*Abcc1*<sup>-/-</sup>) on a FVB genetic  
122 background were adrenalectomized and infused with corticosterone and cortisol by subcutaneous  
123 osmotic minipump for 7 days, followed by measurement of steroids in plasma and tissue by  
124 liquid chromatography tandem mass spectrometry (LC-MS/MS). Plasma analysis (Figure 3A)  
125 indicated impaired clearance of glucocorticoids in *Abcc1*<sup>-/-</sup> mice, with elevated steady state  
126 concentrations of corticosterone and a trend to elevated cortisol compared with control mice, but  
127 there was no difference in the circulating corticosterone:cortisol ratio. In the brain (Figure 3B),  
128 corticosterone concentrations were higher in *Abcc1*<sup>-/-</sup> mice than controls, consistent with  
129 increased plasma concentrations, and cortisol was low in both groups, consistent with exclusion  
130 of cortisol by ABCB1 (10). However, there was no effect of ABCC1 deficiency on brain  
131 corticosterone:cortisol ratio. In contrast, in subcutaneous adipose tissue (Figure 3C), *Abcc1*  
132 deficiency resulted in an elevated corticosterone:cortisol ratio, with a marked increase in  
133 corticosterone but not cortisol concentrations.

134 To assess if pharmacological inhibition of ABCC1 mimicked the effects of genetic deletion on  
135 adipose glucocorticoid concentrations, we treated mice with probenecid. Male C57Bl/6 mice  
136 underwent the same adrenalectomy and steroid infusion protocol as *Abcc1*<sup>+/+</sup> and *Abcc1*<sup>-/-</sup> mice,  
137 and were treated with probenecid (150 mg/kg/day) or vehicle (saline). Probenecid did not alter  
138 corticosterone or cortisol concentrations in the plasma (Figure 3D) or in the brain (Figure 3E);  
139 however, consistent with the results from *Abcc1*<sup>-/-</sup> mice, probenecid increased corticosterone but  
140 not cortisol concentrations in subcutaneous adipose tissue (Figure 3F).

141 To test the metabolic consequences of the increase in intra-adipose corticosterone concentrations  
142 with ABCC1 inhibition, we repeated the experiment with probenecid, adding an additional  
143 control group with no glucocorticoid infusion, and assessed adipose tissue for glucocorticoid-  
144 responsive gene transcripts (Figure 4). Plasma corticosterone and cortisol concentrations were  
145 unaffected by probenecid in corticosteroid-infused groups, as before (Figure 4A), and below the  
146 lower limit of detection (0.5 ng/ml) in vehicle-treated adrenalectomized animals. Corticosteroid  
147 infusion reduced weight gain during the 7-day treatment (Figure 4B) and induced adipose  
148 expression of *Per1*, *Atgl*, *Hsl*, and *Lpl* (Figure 4C); the effects on body weight and adipose *Per1*,  
149 *Atgl*, and *Lpl* were potentiated by probenecid (Figure 4B,C).

150 *Corticosterone concentrations are low in human adipose, and not increased in obesity*

151 To compare endogenous cortisol and corticosterone concentrations in adipose tissue, and to test  
152 whether downregulation of ABCC1 might enhance corticosterone action in adipose tissue in  
153 obesity, we collected subcutaneous and visceral adipose biopsies during elective abdominal  
154 surgical procedures from 6 lean and 6 obese patients, with characteristics shown in table S1.  
155 *ABCC1* mRNA transcripts were increased in obese subjects in both subcutaneous and visceral  
156 adipose (Figure 5A). Adipose tissue cortisol was readily measured in both adipose depots, as  
157 previously described (20), and not different between lean and obese subjects (Figure 5B).  
158 Corticosterone concentrations in lean and obese subjects were below or near the limit of  
159 detection by LC/MS-MS in both visceral and subcutaneous adipose tissue (Figure 5B).

160 *Human adipose is less sensitive to corticosterone than cortisol*

161 Having established that selective expression of ABCC1 protects human adipose tissue from  
162 corticosterone, we then tested whether corticosterone is less potent than cortisol at inducing  
163 metabolic effects in adipose. This was achieved using doses of cortisol and corticosterone

164 equipotent for ACTH suppression. We performed a randomized single-blind crossover study in  
165 patients with Addison's disease using sequentially increasing steady state infusions of deuterated  
166 glucocorticoids, D8-corticosterone and D4-cortisol, which can be distinguished from residual  
167 endogenous glucocorticoids by mass spectrometry (21). Characteristics of study participants are  
168 summarized in table S2. Circulating concentrations of cortisol (sum of cortisol, D4-cortisol, and  
169 its active metabolite D3-cortisol) and corticosterone (D8-corticosterone alone; endogenous  
170 corticosterone was undetectable) indicated that serially increasing steady state concentrations  
171 were achieved as intended (Figure 6A). Total steady state concentrations tended to be higher for  
172 cortisol than corticosterone, although the differences were not statistically significant and there  
173 were no differences in free cortisol and corticosterone measured after equilibrium dialysis (in  
174 samples pooled between 260 and 330 min of infusion, free cortisol =  $38.7 \pm 8.2$  nM and free  
175 corticosterone =  $37.2 \pm 4.7$  nM). Neither ACTH concentrations at baseline nor their suppression  
176 during glucocorticoid infusion were different between cortisol and corticosterone (Figure 6B,C;  
177 Table 1). Amongst biochemical markers of glucocorticoid action – glucose, insulin, glycerol, and  
178 free fatty acids – only free fatty acids rose during steroid infusion (Table 1), perhaps because the  
179 infusions were short and the glucocorticoid concentrations achieved were within the  
180 physiological range (22). Insulin concentrations fell, paradoxically, during steroid infusions.  
181 None of these plasma biochemical markers differed between D8-corticosterone and D4-cortisol  
182 infusion (Table 1). However, in subcutaneous adipose biopsies obtained at the end of infusions,  
183 the acutely responsive glucocorticoid-responsive transcript *PER1* was substantially higher and  
184 *LPL* also modestly higher after D4-cortisol than D8-corticosterone infusion (Figure 6D).

185 **Discussion**

186 Our findings shed light on tissue-specific responsiveness to cortisol and the often-neglected  
187 'second' glucocorticoid corticosterone, mediated by local expression of steroid-selective ABC  
188 transporters. In combination with tissue-specific expression of either mineralocorticoid or  
189 glucocorticoid receptors and tissue-specific intracellular metabolism of corticosteroids, the  
190 availability of alternate endogenous glucocorticoids that are differentially transported out of  
191 target cells provides a mechanism for subtle control of the otherwise highly conserved pathway  
192 of glucocorticoid action. Moreover, as summarized in the schematic in Figure 7, our data show  
193 how this insight can be exploited to develop corticosterone as a potentially safer alternative to  
194 cortisol for glucocorticoid replacement therapy when ACTH suppression is desirable.

195 For in vitro studies, we used SGBS adipocytes, an established model of human  
196 adipocytes (14), to show that in the absence of ABCB1, endogenous ABCC1 expression is  
197 sufficient to reduce intracellular corticosterone but not cortisol concentrations. An equilibrium in  
198 intracellular cortisol and corticosterone concentrations was reached after approximately 4 hours  
199 in adipocytes. Our CARS microscopy data suggest that glucocorticoids co-localize with lipid  
200 droplets within adipocytes, potentially resulting in their intracellular retention and explaining the  
201 slow turnover of cortisol in adipose tissue in vivo (20). The magnitude of the effect of ABCC1  
202 on steroid export appears to be substantial, with a 3-4 fold increase in intracellular corticosterone  
203 when ABCC1 is inhibited in SGBS cells, and a 2-fold increase in adipose corticosterone in vivo  
204 in mice. In a physiological context, if the plasma glucocorticoid pool is comprised of 90-95%

205 cortisol and 5-10% corticosterone, as reported in previous studies (10, 13, 23-32) (table S3), the  
206 total (cortisol plus corticosterone) intra-adipose glucocorticoid pool may be reduced by as much  
207 as ~7% as a result of ABCC1 activity. In a pathological context, we hypothesized that reduction  
208 of adipose ABCC1 in obesity might contribute to metabolic dysregulation through increased  
209 intra-adipose corticosterone. However, *ABCC1* expression was increased in obesity, suggesting a  
210 potential protective mechanism to limit adipose exposure to glucocorticoids, which is considered  
211 to be beneficial in obesity (3). Indeed, adipose glucocorticoid concentrations were consistent  
212 with ABCC1 acting as constitutive barrier to maintain very low corticosterone concentrations in  
213 human adipose tissue, even in obese individuals. In a pharmacological context, such as if  
214 corticosterone were used as glucocorticoid replacement therapy, the consequences of ABCC1  
215 activity for the intra-adipose glucocorticoid pool could be much greater. We tested this in vivo in  
216 mice and humans.

217 Our findings in vivo support our cell-based work, with both genetic deletion and  
218 pharmacological inhibition of ABCC1 resulting in preferential accumulation of corticosterone  
219 over cortisol in the adipose tissue. Because 17 $\alpha$ -hydroxylase, which is necessary to produce  
220 cortisol, is not expressed in mouse adrenals, we studied this in adrenalectomized mice infused  
221 with equal concentrations of both glucocorticoids. Plasma steroid concentrations achieved during  
222 infusion were similar within strains, but slightly higher in C57Bl6 than FVB mice, potentially  
223 reflecting strain-specific differences in glucocorticoid clearance. *Abcc1*<sup>-/-</sup> mice, but not mice  
224 treated with probenecid, showed higher steady-state plasma concentrations of cortisol and  
225 corticosterone compared to wild type controls, implicating ABCC1 in total glucocorticoid  
226 clearance; this effect, however, appears to require total loss of *ABCC1*, as it was not observed  
227 with a competitive inhibitor, probenecid. Of note, the only spontaneous phenotype reported in

228 *Abcc1*<sup>-/-</sup> mice is a reduced susceptibility to inflammation (33), which may be mediated by  
229 systemic or intracellular corticosterone excess, but this has not been studied. Using probenecid,  
230 we showed that the increase in intra-adipose corticosterone concentrations with ABCC1  
231 inhibition is sufficient to potentiate the induction of key lipolytic genes (*Atgl* and *Hsl*) and  
232 prevent weight gain.

233 To investigate the physiological and therapeutic implications of these findings, we  
234 performed a study in patients with Addison's disease, who lack endogenous glucocorticoids. We  
235 selected patients with Addison's disease rather than with CAH to avoid confounding effects of  
236 high androgen concentrations in the adipose tissue. Corticosterone is only available as an  
237 experimental tool for infusion and not as a licensed pharmaceutical, therefore only short-term  
238 manipulation was possible. At similar concentrations of free cortisol and corticosterone, we  
239 found equipotent suppression of ACTH, but substantially greater gene transcript induction in  
240 adipose tissue by cortisol. In adipose, cortisol induced greater transcript expression than  
241 corticosterone not only for *PER1*, which is known to be rapidly induced by glucocorticoids (18)  
242 and sensitive at low concentrations which do not alter other glucocorticoid-responsive genes  
243 (19), but also for *LPL*, a key enzyme involved in adipose triglyceride uptake. The lack of  
244 differences in circulating metabolic markers is likely attributable to the short duration of  
245 infusions and the glucocorticoid concentrations within the physiological range (22); previous  
246 studies have that demonstrated plasma glucose, insulin, and glycerol show responses only to  
247 several hours of 'physiological' glucocorticoid infusion (34, 35). It remains possible that there  
248 are intrinsic differences in transcriptional response to cortisol and corticosterone in human  
249 adipose tissue, even when the same concentrations of steroid are present, but this seems unlikely  
250 given our findings in SGBS adipocytes that cortisol is no more potent than corticosterone when

251 ABCC1 is inhibited. Despite our confirmation that cortisol concentrations are disproportionately  
252 low in the mouse brain, and earlier observations of relatively low cortisol in human brain and  
253 cerebral spinal fluid (10, 13), we did not find that corticosterone was more effective than cortisol  
254 in suppressing ACTH. This may reflect the major feedback signal being mediated in the pituitary  
255 rather than higher centers within the brain, under non-stressed conditions. Nonetheless, the  
256 discrepancy between suppression of ACTH and induction of adipose *PER1* and *LPL* by cortisol  
257 versus corticosterone supports our interpretation that adipose tissue is physiologically more  
258 responsive to cortisol than corticosterone, and that this can be exploited therapeutically.

259 Synthetic glucocorticoids in common use as glucocorticoid therapy, specifically  
260 prednisolone and dexamethasone, are not transported by ABCC1 (11) and so are likely to access  
261 adipose tissue similarly to cortisol. To validate and exploit these findings, it will therefore be  
262 important to develop a suitable pharmaceutical preparation of corticosterone. With longer term  
263 therapy, corticosterone and cortisol effects can be compared in conditions requiring effective  
264 ACTH suppression such as CAH, Nelson's syndrome, and glucocorticoid-suppressible  
265 hyperaldosteronism. Although we have focused here on adipose tissue, differential expression of  
266 ABCB1 and ABCC1 may determine tissue-specific responses to corticosterone and cortisol in  
267 other tissues as well. Adverse effects will need to be compared not only for metabolism and  
268 obesity, but also for immune suppression and osteoporosis. Nevertheless, our findings suggest  
269 that the substantial investment required to develop a corticosterone-based drug product is  
270 worthwhile.

271



272 **Materials and Methods**

273 *Study design*

274 To investigate the role of adipose ABCC1 regulating cortisol versus corticosterone action, we  
275 conducted studies in cells, mice, and humans, as detailed below. In vitro experiments were  
276 performed in triplicate with the number of experiments and outcomes defined in figure legends.  
277 For in vivo studies in mice, experiments were approved by the institutional ethical committee  
278 and conducted under UK Home Office license in male mice aged 10-12 weeks at the start of the  
279 experiment. Sample sizes were chosen for 80% power to detect magnitudes of difference inferred  
280 from in vitro experiments at  $P < 0.05$ , with the number of mice and outcomes defined in figure  
281 legends. Mice of each genotype were randomly assigned to interventions within each  
282 experiment. The studies in humans were conducted with approval from the South East Scotland  
283 Research Ethics Committee and NHS Lothian Research and Development (13/SS/0210 for  
284 Addison's disease study and 10/S1102/39 for surgical adipose tissue collection study), and with  
285 written informed consent of participants. The surgical adipose tissue collection study was a case-  
286 control design comparing adipose tissue mRNA amounts in lean and obese participants randomly  
287 selected from those undergoing surgery. An initial exploratory study was conducted in  $n=6$  per  
288 group and showed results contradicting the hypothesis that *ABCC1* transcripts are decreased in  
289 obesity, so the study was not expanded. For the Addison's disease study, eligible patients  
290 participated in a single-blind randomized crossover study comparing infusions of deuterated  
291 corticosterone and deuterated cortisol for effects on ACTH, adipose tissue mRNA, and  
292 circulating metabolic indices. Blinding of the clinical investigator was impractical because of the

293 requirements for administration of different loading and infusion doses of cortisol and  
294 corticosterone, but samples were processed in the laboratory with blinded codes and only  
295 decoded for statistical analysis; patients were blinded to treatment. Sample size was calculated  
296 for 90% power to detect 20% difference in ACTH at  $p < 0.05$ . Inclusion/exclusion criteria for each  
297 study are detailed below. There were no dropouts, and no outliers were excluded from any  
298 studies.

### 299 *Human adipose tissue microarray*

300 Affymetrix microarray data were obtained from subcutaneous adipose biopsies of 9 healthy men,  
301 aged  $36.6 \pm 2.1$  years with body mass indices  $29.2 \pm 2.1$  kg/m<sup>2</sup> (36). Probes for transcripts  
302 encoding ABC transporters were selected and ranked by transcript intensity ( $\log^2$  transformed).

### 303 *Polymerase chain reaction*

304 Human tissue biobank cDNA (Primerdesign Ltd) was used as a template for the identification of  
305 ABC transporters in human tissue of interest. Equine peri-renal adipose tissue collection from  
306 clinical cases euthanized for reasons other than endocrine disease/systemic inflammation was  
307 approved by the University of Edinburgh Veterinary ethics and research committee. Where  
308 applicable, total RNA extraction was carried out by centrifugation in Qiazol lysis reagent using  
309 an RNeasy mini kit (Qiagen), according to the manufacturer's instructions. RNA integrity was  
310 checked on a 1% agarose gel, after which cDNA (500 ng RNA/reaction) was synthesized using a  
311 high capacity cDNA kit (Invitrogen), according to the manufacturer's instructions. cDNA was  
312 used as a template for specific primers (Invitrogen; table S4) for RT-PCR and amplified using  
313 GoTaq DNA polymerase (Promega) as per the manufacturer's instructions, on a Techne  
314 thermocycler (95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; 72 °C  
315 for 5 min). Products were subjected to gel electrophoresis on a 2% agarose gel in TAE buffer

316 (50x stock buffer: 2 M Tris Base, 1 M glacial acetic acid, 100 mM disodium EDTA) containing  
317 GelRed (Cambridge Bioscience). Gels were imaged using a Gel-Doc system (Uvitec). Product  
318 size was confirmed against a 100 bp DNA ladder (Invitrogen).

## 319 **In vitro studies**

### 320 *SGBS cell culture*

321 Human preadipocyte Simpson-Golabi-Behmel syndrome (SGBS) cells were a kind gift from  
322 Martin Wabitsch, University of Ulm, Germany (14). Cells were maintained in high-glucose  
323 DMEM-F12 (Lonza), supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin  
324 (100 IU/ml), biotin (33  $\mu$ M), and pantothenic acid (17  $\mu$ M) at 37°C in 5% CO<sub>2</sub>. Differentiation  
325 was induced as previously described (37). Cells were cultured in steroid-free medium for 24  
326 hours before experimentation.

### 327 *Primary cell culture*

328 Subcutaneous adipose tissue samples were obtained from patients undergoing elective abdominal  
329 surgery at the Royal Infirmary of Edinburgh. Upon tissue collection, adipose was digested, and  
330 the stromal vascular fraction was isolated and differentiated as previously described (38). In  
331 brief, after removal of connective tissue and blood vessels, adipose tissue was digested in  
332 collagenase type I (615 U/g tissue, 90 min, 37 °C). After overnight plating in high-glucose  
333 DMEM-F12 supplemented with FBS (10% v/v), penicillin (100 IU/ml), streptomycin (100  
334 IU/ml), biotin (33  $\mu$ M), and pantothenic acid (17  $\mu$ M), cells were differentiated for 3 days using  
335 the above medium without serum, but with addition of triiodothyronine (1 nM), transferrin (10  
336  $\mu$ g/ml), insulin (66 nM), IBMX (500  $\mu$ M), dexamethasone (1  $\mu$ M), and rosiglitazone (10  $\mu$ M).  
337 From day 4 onwards, cells were maintained in this differentiation medium but without IBMX,  
338 dexamethasone, or rosiglitazone.

339 *Tritiated glucocorticoid retention assays*

340 To measure uptake and retention of steroids, SGBS adipocytes were incubated in the presence of  
341 either corticosterone or cortisol (20 nM 1,2,6,7-<sup>3</sup>H<sub>4</sub>-steroid, 480 nM unlabeled steroid) for 1, 4,  
342 8, and 24 hours before being washed in PBS, lysed in cellular lysis buffer (0.5% SDS), mixed  
343 with Prosafe FC+ liquid scintillation cocktail (Meridan Biotechnologies), and read for 1 min on a  
344 β-scintillation counter. A separate batch of SGBS adipocytes were incubated with steroids for 24  
345 hours as described above, then washed in PBS before fresh steroid-free medium was added to  
346 each well and the decline in intracellular <sup>3</sup>H-steroid content measured at 1, 4, 8 and 24 hours.

347 To assess the effect of ABCC1 inhibition, SGBS adipocytes were pre-incubated for 1 hour in the  
348 presence of the indicated concentrations of MK-571 (Cayman Chemical), probenecid  
349 (Invitrogen), or vehicle (DMSO), before being incubated for a further 24 hours in the presence of  
350 inhibitor or vehicle and either corticosterone or cortisol (20 nM <sup>3</sup>H-steroid, 480 nM unlabeled  
351 steroid). Cells were then washed in PBS, lysed in cellular lysis buffer, mixed with liquid  
352 scintillation cocktail, and read for 5 min on a β-scintillation counter.

353

354 *Quantification of glucocorticoid-sensitive mRNA transcripts*

355 In accordance with the time course of the clinical study, we performed an acute (2 hour)  
356 treatment in SGBS adipocytes and an extended (24 hour) treatment with either vehicle or cortisol  
357 (500 nM) to identify acutely upregulated glucocorticoid-responsive transcripts. To determine the  
358 effects of ABCC1 inhibition on glucocorticoid-induced transcripts, SGBS adipocytes were pre-  
359 incubated for 1 hour in the presence of probenecid (50 μM) or vehicle (DMSO), before a further  
360 2 or 24 hours incubation in the presence of probenecid or vehicle and either corticosterone or  
361 cortisol (500 nM). Total RNA was extracted from adipocytes in Qiazol lysis reagent using a

362 RNeasy Mini Kit according to the manufacturer's instructions. RNA (250 ng) was reverse  
363 transcribed with random primers using the Applied Biosystems High-Capacity cDNA Reverse  
364 Transcription kit. Real-time PCR was performed using the Roche LightCycler 480 (Roche  
365 Applied Science). Primers (Invitrogen) were designed for use with intron-spanning probes within  
366 the Roche Universal Probe Library (UPL). Primer sequences and UPL probe numbers are shown  
367 in table S5. Results were corrected for abundance of 18S, which was not affected by treatment.  
368 All samples were analyzed in triplicate and amplification curves plotted (y axis fluorescence, x  
369 axis cycle number). Triplicates were deemed acceptable if the standard deviation of the crossing  
370 point was < 0.5 cycles. A standard curve (y axis crossing point, x axis log concentration) for each  
371 gene was generated by serial dilution of cDNAs pooled from different samples, fitted with a  
372 straight line, and deemed acceptable if reaction efficiency was between 1.7 and 2.1.

### 373 *CARS microscopy*

374 Coherent Anti-Stokes Raman Scattering (CARS) microscopy is a non-invasive, label-free  
375 imaging technique based on Raman spectroscopy. The experimental setup has been described  
376 previously (39). Briefly, a pump and tuneable Stokes laser (PicoTrain, High-Q laser and Levante  
377 Emerald Optical Parametric Oscillator) provided a specific vibrational coherence resulting in  
378 detectable photons which, when combined with a confocal laser scanning inverted microscope  
379 (C1 Eclipse, Nikon BV), provided a spatial image. For CARS imaging, the pump and Stokes  
380 laser beams were tuned such that the frequency difference would correspond to a specific Raman  
381 vibration.

382 SGBS adipocytes were incubated for 24 hours in glass-bottomed dishes with  
383 2,2,4,6,6,17 $\alpha$ ,21,21-<sup>2</sup>H<sub>8</sub>-corticosterone (D8-corticosterone, 10  $\mu$ M; Cambridge Isotopes) in the  
384 presence or absence of MK-571 (10  $\mu$ M). For CARS imaging, the pump laser was tuned to 816.8

385 nm ( $12243\text{ cm}^{-1}$ ) and Stokes laser to 1064 nm ( $9398.5\text{ cm}^{-1}$ ) to obtain a CARS signal at 663 nm  
386 ( $2845\text{ cm}^{-1}$ ) corresponding to the vibration of  $\text{CH}_2$  in lipids (39). After initial documentation of  
387 Raman spectra to identify optimal wavelength (figure S5), excitation of the Raman vibration of  
388 the C-D bond in D8-corticosterone was achieved by adjusting the pump laser to 868.4 nm  
389 ( $11524.5\text{ cm}^{-1}$ ), resulting in a CARS signal at 733 nm ( $2126\text{ cm}^{-1}$ ), which was in a region of the  
390 Raman spectrum with low background signal. Images were processed using Nikon EZ-C1 3.4  
391 software.

### 392 *Protein analysis*

393 Total protein extracts from cells were prepared in RIPA buffer (Santa Cruz). Samples were  
394 sonicated and centrifuged ( $13,000 \times g$ , 15 min,  $4\text{ }^\circ\text{C}$ ) before performing the BCA assay, adding  
395 6X Laemmli sample buffer (LSB), boiling, and SDS-PAGE resolution. ABCC1 (Enzo Life  
396 Sciences, ALX-801-007) and HSP90 (Santa Cruz, sc-7947) were detected on Western blots  
397 using commercial antibodies.

398

### 399 *Oil Red O staining and lipid quantification*

400 To assess potential differing effects of cortisol and corticosterone on adipocyte differentiation,  
401 SGBS adipocytes were stimulated to undergo differentiation with or without the substitution of  
402 cortisol in the differentiation cocktail (37) for corticosterone. Accumulation of lipid during  
403 adipogenesis was visualized by Oil Red O (Sigma-Aldrich). Cells were washed twice with PBS  
404 and fixed with formalin (10% v/v) for 60 min, followed by a wash with isopropanol (60% v/v).  
405 Working Oil Red O solution was added to cells for 10 min, followed by 4 washes with  $\text{H}_2\text{O}$ .  
406 Cells were air-dried and dye extracted with isopropanol (100%). Absorbance of extracts were  
407 measured at 500 nm wavelength in an OPTImax microplate reader (Molecular Devices).

408 *Calcein-AM Assay*

409 Calcein–acetoxymethyl ester (AM) is a non-fluorescent, hydrophilic, cell membrane permeable  
410 molecule which is converted to fluorescent, hydrophilic calcein by intracellular esterases (16).  
411 Both calcein-AM and calcein are substrates for ABCC1, thus intracellular fluorescence is  
412 inversely proportional to ABCC1 activity. SGBS adipocytes were washed with PBS and pre-  
413 incubated for 1 hour in the presence of MK-571 (10  $\mu$ M) or vehicle (DMSO), before being  
414 incubated for a further 5 hours in the presence of inhibitor or vehicle and calcein-AM (1  $\mu$ M;  
415 Invitrogen). Cells were washed three times in PBS, then excitation absorbance was measured at  
416 494 nm and emission absorbance was measured at 517 nm in an Infinite M1000 plate reader  
417 (Tecan). Data are expressed as percentage fluorescence of untreated control.

418 *Luciferase reporter assay*

419 Human epithelial A549 cells were grown in DMEM (Lonza), supplemented with FBS (10% v/v),  
420 penicillin (100 IU/ml), and streptomycin (100 IU/ml). Cells were seeded at  $2 \times 10^5$ / 35 mm well.  
421 After overnight incubation, medium was replaced with Opti-MEM (Lonza), and cells were  
422 transfected using Lipofectamine 2000 (Invitrogen) with a total of 2  $\mu$ g DNA comprising 1  $\mu$ g  
423 pMMTV-LTR-Luc (40) and 1  $\mu$ g pKC275 (encoding  $\beta$ -galactosidase as internal control). After  
424 overnight incubation, medium was replaced with steroid-free medium, and cells were treated for  
425 1 hour with MK-571 (10  $\mu$ M) before a 24-hour incubation with inhibitor and either  
426 corticosterone or cortisol (500 nM). Luciferase and  $\beta$ -galactosidase activities were measured in  
427 cell lysates as described previously (41).  $\beta$ -Galactosidase activity was assayed using a Tropic  
428 Galacto Light Plus kit (Applied Biosystems). All transfections were carried out in triplicate, and  
429 the mean ratio of luciferase/  $\beta$ -galactosidase activities was calculated.

430 **Animal studies**

431 *Animals*

432 Male *Abcc1* knockout mice (*Abcc1*<sup>-/-</sup>) and FVB controls (*Abcc1*<sup>+/+</sup>) were purchased from  
433 Taconic. Male C57Bl/J mice were purchased from Charles River. Mice were bilaterally  
434 adrenalectomized under fluorothane anesthesia. After surgery, drinking water was replaced with  
435 0.9% NaCl, and animals were allowed to recover for 7 days. An osmotic mini-pump (Alzet  
436 Model 2001; 1 µl/hr) delivering 250 µg/day of corticosterone and 250 µg/day cortisol or vehicle  
437 (DMSO: propylene glycol; 50:50 v/v) was inserted subcutaneously under anesthesia and left in  
438 place for 7 days. *Abcc1*<sup>+/+</sup> and *Abcc1*<sup>-/-</sup> mice received no further treatment. C57Bl/6 mice were  
439 given daily s.c. injections of probenecid (150 mg/kg) or vehicle (saline). This dose was  
440 previously reported to inhibit transporter activity in vivo (42). 7 days after osmotic pump  
441 implantation, animals were culled by decapitation. Plasma was extracted from trunk blood and  
442 stored at -20 °C. Tissue was extracted and stored at -80 °C.

443

444 *Plasma and tissue steroid extraction and LC-MS/MS quantification*

445 Steroids were extracted from plasma by liquid-liquid extraction (chloroform, 10:1). Briefly,  
446 plasma (100 µl) was enriched with internal standard (D4-cortisol and epi-corticosterone, 25 ng  
447 each). 1 ml chloroform was added and vortexed. Supernatant was reduced to dryness under  
448 oxygen-free nitrogen (OFN) at 60°C and reconstituted in mobile phase [70 µl water/acetonitrile  
449 (70:30, v/v)]. Steroids were extracted from brain and adipose as previously described (20), with  
450 the substitution of D4-cortisol and D8-corticosterone as internal standards. Steroids were  
451 extracted from brain by homogenizing whole brain in methanol:acetic acid (100:1 v/v; 10 ml)  
452 and centrifuging (5000x g, 10 min, 4°C). The supernatant was reduced to dryness under OFN at



453 60°C, reconstituted in methanol:dichloromethane:water (7:2:1, v/v; 3 ml), and enriched with  
454 internal standard (D4-cortisol and D8-corticosterone, 25 ng each). Samples were passed through  
455 a diethylaminohydroxypropyl Sephadex LH-20 anion exchange column (GE Healthcare).  
456 Columns were washed with methanol:dichloromethane:water (7:2:1, v/v; 2 ml) and  
457 methanol:dichloromethane:water (2:2:1, v/v; 1 ml). All flow-through/wash was collected and  
458 reduced to dryness under OFN at 60°C. Samples were reconstituted in methanol:acetic acid  
459 (100:1 v/v; 2 ml), and water (2 ml) was added. Samples were passed through pre-conditioned C-  
460 18 Bond-Elut columns (Agilent Technologies). Columns were washed with water (2 ml),  
461 methanol:water (50:50 v/v; 3 ml), and hexane:ethyl acetate (5:1 v/v; 2 ml). Samples were eluted  
462 in ethyl acetate (2 ml), dried under OFN at 60°C and reconstituted in mobile phase [60 µl  
463 water/acetonitrile (70:30, v/v)].

464 Quantitative analysis of steroids was carried out by liquid chromatography tandem mass  
465 spectrometry (LC-MS/MS). Chromatographic separation was achieved using a Waters Acquity  
466 UPLC system, with detection on an ABSciex QTRAP 5500 mass spectrometer operated with  
467 Analyst software version 1.6.1. The mass spectrometer was operated using the Turbospray Ion  
468 source, with nitrogen as the source, curtain and collision gas (40 and 60 psi, medium), source  
469 temperature of 550°C, spray voltage of 4.5 kV, and an entrance potential of 10 V. Compound-  
470 specific tuning was performed using methanolic solutions of steroids and isotopically labeled  
471 internal standards. The protonated molecular ions were subjected to collision-induced  
472 dissociation, and the most abundant precursor-product transitions were selected;  $m/z$  347.2 →  
473 91.1, 121.1 at 69 V,  $m/z$  355.1 → 337.0 at 19 V,  $m/z$  363.2 → 121.0, 77.0 at 29 and 101 V, and  
474  $m/z$  367.0 → 121.1 at 29 and 101 V collision energy for corticosterone, D8-corticosterone,  
475 cortisol, and D4-cortisol, respectively. Analytes were eluted on a Waters Sunfire C18 column

476 (150 x 2.1 mm; 3.5 µm) at 30°C (injection volume; 30 µL) protected by a Kinetex KrudKatcher  
477 at a flow rate of 0.5 mL/min, starting at 30:70 and rising linearly to 90:10 [water+0.1% formic  
478 acid (FA):acetonitrile+0.1% FA] by 6 minutes and a total run time, including re-equilibration, of  
479 9 minutes. The peak areas were integrated using Xcalibur software (Thermo Electron) and  
480 quantified against a calibration curve. Steroid concentrations are presented corrected for total  
481 tissue weight or plasma volume.

#### 482 *Quantification of glucocorticoid-sensitive mRNA transcripts*

483 RNA analysis performed was as described above. Primer sequences and UPL probe numbers are  
484 shown in table S6. Results were corrected for abundance of the mean combination of *18S* and  
485 *Tbp*, which was not affected by treatment.

486

#### 487 **Adipose tissue steroid concentrations and *ABCC1* mRNA in lean and obese humans**

488 We recruited 12 subjects who were at the Royal Infirmary of Edinburgh for elective abdominal  
489 surgery for non-malignant disease, and who did not have systemic or local active inflammation.  
490 Paired adipose tissue samples were obtained intra-operatively from the subcutaneous and  
491 visceral depots, stored on dry ice then at -80C, and extracted for steroid quantification as  
492 previously described (20) and for RNA analysis as described above.

493

#### 494 **Cortisol versus corticosterone infusion in patients with Addison's disease**

##### 495 *Participants*

496 Patients with Addison's disease were identified from the clinic database of the Edinburgh Centre  
497 for Endocrinology and invited to attend a screening visit, where written informed consent was  
498 obtained and eligibility assessed by a medical questionnaire, physical examination, and routine

499 blood tests. Inclusion criteria were: a diagnosis of autoimmune Addison's disease and age >18 y.  
500 Exclusion criteria were: alcohol intake >28 units/week; abnormal screening blood results (full  
501 blood count; renal, liver, and thyroid function tests); pituitary disease; pregnant or breastfeeding;  
502 anti-inflammatory glucocorticoid therapy by any route in the preceding 3 months; cardiac, renal  
503 or liver failure; uncontrolled hypertension (systolic BP >160 mmHg or diastolic >100 mmHg);  
504 blood donation in preceding 3 months (to avoid anemia); or research study participation in  
505 preceding 6 weeks.

#### 506 *Study protocol*

507 Participants attended for study visits on two occasions, separated by at least one week.  
508 Participants withheld hydrocortisone from 14:00h on the day before each study visit, and omitted  
509 fludrocortisone (when prescribed) the morning before the study visit and on the morning of the  
510 study visit. They attended the clinical research facility at 08:00h after an overnight fast from  
511 22:00h. At  $t = -15$  minutes, intravenous cannulae (18G) were inserted in each antecubital fossa.  
512 At  $t = 0$ , saline infusion (0.9 %, 125 ml/h) was commenced through the cannula in the left arm,  
513 and blood samples collected from the cannula in the right arm. At  $t = 60$  min, infusion of  
514 deuterated glucocorticoid (D8-corticosterone or D4-cortisol) was commenced. Order of steroid  
515 infusion was allocated randomly, and study participants were blinded to the order of infusate.  
516 9,11,12,12- $^{2}\text{H}$ <sub>4</sub>-cortisol (D4-cortisol) and 2,2,4,6,6,17,21,21- $^{2}\text{H}$ <sub>8</sub>-corticosterone (D8-  
517 corticosterone) were obtained from Cambridge Isotope Laboratories, dissolved in pharmaceutical  
518 grade ethanol/water (90:10, v/v) and filtered to form sterile stock solutions, stored (-40 °C) for a  
519 maximum of 8 weeks. On study days, D8-corticosterone (4.18 mg/ml) or D4-cortisol (2.5  
520 mg/ml) stock solution (5 ml) was dissolved in sodium chloride 0.9% w/v (495 ml). At  $t = 60$  min,  
521 a priming dose (0.65  $\mu\text{mol}$  D8-corticosterone; 0.23  $\mu\text{mol}$  D4-cortisol) was administered over 4

522 minutes, followed by steady state infusion (27.6 nmol/min D8-corticosterone; 3.7 nmol/min D4-  
523 cortisol) for 86 minutes. Further priming doses were administered at  $t = 150$  min (1.95  $\mu$ mol D8-  
524 corticosterone; 0.65  $\mu$ mol D4-cortisol) followed by constant infusion (111.2 nmol/min D8-  
525 corticosterone; 17.2 nmol/min D4-cortisol) until  $t = 240$  min, and at 240 min (3.89  $\mu$ mol D8-  
526 corticosterone; 1.55  $\mu$ mol D4-cortisol) followed by constant infusion (277.8 nmol/min D8-  
527 corticosterone; 51.5 nmol/min D4-cortisol) until  $t = 330$  min.

528 At  $t = 330$  min, a needle aspiration biopsy of subcutaneous abdominal adipose tissue was  
529 obtained as previously described (36) and stored at  $-80$  °C.

530 Blood samples were obtained at 10-20 min intervals in potassium EDTA tubes (2.7 ml) pre-  
531 chilled on wet-ice and serum gel tubes (9 ml; both Monovette, Sarstedt). Potassium EDTA tubes  
532 were centrifuged at  $4$  °C within 30 minutes of sampling; serum gel samples were left at room  
533 temperature for 30-45 minutes before centrifugation. Serum and plasma was stored at  $-80$  °C.

#### 534 *Laboratory analyses*

535 In plasma, ACTH was quantified by ELISA (IBL International) within 6 weeks of sampling, and  
536 non-esterified fatty acids (NEFAs)(Zen-Bio) and glycerol (Sigma-Aldrich) quantified by  
537 colorimetric assays. In serum, glucocorticoids were quantified by LC-MS/MS, insulin by ELISA  
538 (DRG Diagnostics), and glucose by colorimetric assay (Cayman Chemical).

539 LC-MS/MS analysis was undertaken as described for animal samples above with the exception  
540 that  $11\alpha$ -epimers of corticosterone (epi-corticosterone;  $m/z$  347.2  $\rightarrow$  91.1, 121.1 at 69 V) and  
541 cortisol (epi-cortisol; ( $m/z$  363.2  $\rightarrow$  121.0, 77.0 at 29 and 101 V) were used as internal standards  
542 instead of D8-corticosterone and D4-cortisol.

543 To account for any differences in protein binding between cortisol and corticosterone, cortisol  
544 and corticosterone isotopologs were also measured by LC-MS/MS after equilibrium dialysis of

545 plasma. To achieve the necessary sensitivity, samples were pooled for the final steady state  
546 period from each infusion (260-330 min), and 4 x 1 mL aliquots of plasma were dialyzed into 1.5  
547 mL phosphate buffered saline across 12-14 kD dialysis membrane (Medicell) for 16 hours at 37  
548 °C, as previously described (43), before LC-MS/MS analysis of the pooled dialysate as above.  
549 Real-time qPCR was carried out in adipose tissue as with SGBS cells above.

## 550 **Statistical analysis**

551 For cell and mouse-based studies, comparisons were performed using two-way ANOVA with  
552 Bonferroni post-hoc tests or unpaired Student's t tests as outlined in each figure legend. For the  
553 Addison's disease study, data from each steady state period (80-140 min; 160-240 min; 260-330  
554 min) were averaged, and comparisons between D4-cortisol and D8-corticosterone and the  
555 interaction with changes over time were performed by two-way ANOVA. Adipose tissue data  
556 were compared with paired Student's t tests. P values for statistically significant differences are  
557 presented in table S7.

558 **Supplementary Materials**

559 *Supplementary figures*

560 Fig. S1. Whole gel PCR images of ABC transporter expression in tissues and cells.

561 Fig. S2. Correlation of *ABCC1* mRNA and protein levels in human adipocytes.

562 Fig. S3. ABCC1 inhibition in SGBS adipocytes.

563 Fig. S4. Effects of ABCC1 inhibition on GR-mediated transcription.

564 Fig. S5. Optimisation of CARS microscopy for detection of intracellular D8-corticosterone.

565

566 *Supplementary tables*

567 Table S1. Characteristics of lean and obese study participants providing adipose biopsy samples  
568 during surgery.

569 Table S2. Characteristics of Addison's study participants.

570 Table S3. Summary of studies describing plasma corticosterone and cortisol concentrations in  
571 healthy subjects.

572 Table S4. Primer sequences for PCR and corresponding expected product size.

573 Table S5. Human primer sequences for qPCR and corresponding probe number from Roche  
574 Universal Probe Library (UPL).

575 Table S6. Murine primer sequences for qPCR and corresponding probe number from Roche  
576 Universal Probe Library (UPL).

577 Table S7. Summary table of exact *P* values (provided as an Excel file).

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- 704

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718 contributed to design and execution of the clinical studies. BRW conceived of the studies and  
719 supervised the experimental design, execution and analysis. MN, SDM and BRW wrote the  
720 manuscript which was reviewed by all authors. **Competing interests:** BRW has received an  
721 honorarium for speaking at a symposium sponsored by Shire Pharmaceuticals, who market  
722 Plenadren for treatment of adrenal insufficiency. All other authors declare that they have no  
723 competing interests.

724 **Figures:**

725 **Fig. 1.** *ABCC1*, but not *ABCB1*, is expressed in human adipose tissue. **(A)** Transcript microarray  
 726 data for members of the ABC transporter family, ranked by intensity, in subcutaneous adipose  
 727 tissue from 12 healthy men. *ABCC1* but not *ABCB1* is highly expressed in human adipose.  
 728 Reverse transcriptase polymerase chain reaction amplification (RT-PCR) showing expression of  
 729 *ABCB1* and *ABCC1* in **(B)** human tissues and fully differentiated SGBS human adipocytes, **(C)**  
 730 murine adipose, and **(D)** horse adipose. MNC = mononuclear cells, Sk. Mus = skeletal muscle.

731

732 **Fig. 2.** *ABCC1* preferentially exports corticosterone from adipocytes. **(A)** Export of <sup>3</sup>H-cortisol  
 733 and <sup>3</sup>H-corticosterone from SGBS adipocytes after steroid loading for the previous 24 hours (480  
 734 nM unlabeled steroid + 20 nM tritiated steroid). Data are expressed as a percentage of  
 735 intracellular steroid counts per minute (CPM) after 24 hour loading ( $n = 3$ ,  $*P < 0.05$ ,  $**P < 0.01$ ,  
 736 two-way ANOVA with Bonferroni post-hoc test). **(B)** Coherent anti-Stokes Raman Scattering  
 737 (CARS) images of SGBS cells treated with D8-corticosterone (500 nM) with or without *ABCC1*  
 738 inhibition (MK571, 10  $\mu$ M, 24 hours). Resonance of the C-H bonds abundant in lipid is  
 739 represented in red; C-D resonance from D8-corticosterone is represented in green, and is more  
 740 abundant with MK571 inhibition, notably in a distribution around the intracellular lipid droplets  
 741 (scale bar = 20  $\mu$ m). **(C)** Effect of *ABCC1* inhibition with either MK571 (10  $\mu$ M) or probenecid  
 742 (PBN, 50  $\mu$ M) on <sup>3</sup>H-cortisol and <sup>3</sup>H-corticosterone retention in SGBS adipocytes after 24 hour  
 743 incubation. ( $n = 3$ ,  $***P < 0.001$  vs Vehicle,  $##P < 0.01$ ,  $###P < 0.001$  vs cortisol, two-way  
 744 ANOVA with Bonferroni post-hoc test). **(D)** SGBS adipocytes incubated with corticosterone or

745 cortisol (500 nM, 2 hours) in the presence or absence of probenecid (PBN, 50  $\mu$ M). Transcripts  
746 measured are period circadian clock (*PER1*), adiponectin (*ADIPOQ*), adipose triglyceride lipase  
747 (*ATGL*), and hormone sensitive lipase (*HSL*). ( $n = 3$ ,  $*P < 0.05$  vs Control,  $\#P < 0.05$  vs – PBN,  
748 two-way ANOVA with Bonferroni post-hoc test. **(E)** SGBS pre-adipocytes show greater  
749 triglyceride accumulation after incubation with cortisol than corticosterone (100 nM), quantified  
750 by Oil Red O staining ( $n = 3$ ,  $***P < 0.001$  vs corticosterone, two-way ANOVA with Bonferroni  
751 post-hoc test). All data are mean  $\pm$  SEM, exact  $P$  values are given in table S7.

752  
753 **Fig. 3.** *Abcc1*<sup>-/-</sup> deletion or pharmacological inhibition in mice results in preferential  
754 accumulation of corticosterone in adipose tissue. **(A - C)** Adrenalectomized male wild type  
755 (*Abcc1*<sup>+/+</sup>) or ABCC1 knockout (*Abcc1*<sup>-/-</sup>) FVB mice were infused with corticosterone and  
756 cortisol for 7 days. **(D - E)** Adrenalectomized male C57Bl/6 mice were infused with  
757 corticosterone and cortisol, together with either probenecid or vehicle (saline), for 7 days. Plasma  
758 (A,D), brain (B,E), and subcutaneous adipose (C,F) corticosterone (C'one) and cortisol were  
759 quantified by LC/MS-MS and are presented as concentrations and as corticosterone:cortisol  
760 ratios (Ratio). All data are mean  $\pm$  SEM,  $n = 6-8$  per group, (A - C)  $*P < 0.05$  vs *Abcc1*<sup>+/+</sup>, (D - E)  
761  $*P < 0.05$  vs vehicle, Student's  $t$  test, exact  $P$  values are given in table S7.

762  
763 **Fig. 4.** Pharmacological inhibition of ABCC1 potentiates adipose GC-responsive transcript  
764 expression. **(A)** Plasma corticosterone (C'one) and cortisol concentrations, and their ratio, were  
765 not altered between mice receiving 7 days treatment with corticosteroid (corticosterone and  
766 cortisol 250  $\mu$ g/day) in the presence or absence of probenecid (150 mg/kg/day).  
767 Adrenalectomized mice not receiving corticosteroid infusion had corticosterone or cortisol  
768 concentrations below the detectable limit (0.5 ng/ml). **(B)** Change in body weight of

769 adrenalectomized mice after 7 days of treatment with corticosteroid (steroid) in the presence or  
770 absence of probenecid (PBN), or control (no corticosteroid). (C) Subcutaneous adipose transcript  
771 expression of the glucocorticoid-responsive genes period circadian clock 1 (*Per1*), adipose  
772 triglyceride lipase (*Atgl*), hormone sensitive lipase (*Hsl*), fatty acid synthase (*Fas*),  
773 diacylglycerol O-Acyltransferase 1 (*Dgat1*), and lipoprotein lipase (*Lpl*). All data are mean  $\pm$   
774 SEM ( $n = 7-11$  per group,  $*P < 0.05$  vs control,  $\#P < 0.05$  vs steroid + vehicle, two-way ANOVA  
775 with Bonferroni post-hoc test), exact  $P$  values are given in table S7.

776  
777 **Fig. 5.** Corticosterone concentrations are low in human adipose tissue. Adipose biopsies were  
778 obtained during elective abdominal surgery from 6 lean and 6 obese patients. (A) *ABCC1* mRNA  
779 concentrations were upregulated in obese subcutaneous (SC) and visceral (Visc) adipose tissue.  
780 ( $n = 6$ ,  $*P < 0.05$ ,  $**P < 0.01$  vs lean, unpaired Student's  $t$  tests). (B) Adipose tissue cortisol  
781 concentrations were readily detectable, but corticosterone concentrations were low or below the  
782 limit of detection (LOD) in SC and visceral adipose [samples below limit of detection are  
783 assigned a value of 0.86 pmoles/g (LOD)]. Neither cortisol nor corticosterone differed between  
784 lean and obese participants. All data are mean  $\pm$  SEM, exact  $P$  values are given in table S7.

785  
786 **Fig. 6.** Human adipose tissue is more sensitive to cortisol than corticosterone. A randomized  
787 single blind crossover study with ramped infusion of deuterated cortisol (D4-Cortisol) or  
788 deuterated corticosterone (D8-Corticosterone) was conducted in 9 patients with Addison's  
789 disease. Plasma concentrations of total cortisol and corticosterone (A) or ACTH (B) were not  
790 significantly different throughout the study. (C) Suppression of ACTH was similar during high-  
791 dose infusion of cortisol or corticosterone. (D) Cortisol induced a greater rise in *PER1* and *LPL*



792 mRNA concentrations in subcutaneous adipose tissue (\* $P < 0.05$  vs corticosterone, paired  
793 Student's t-tests). All data are mean  $\pm$  SEM, n=9, exact  $P$  values are given in table S7.

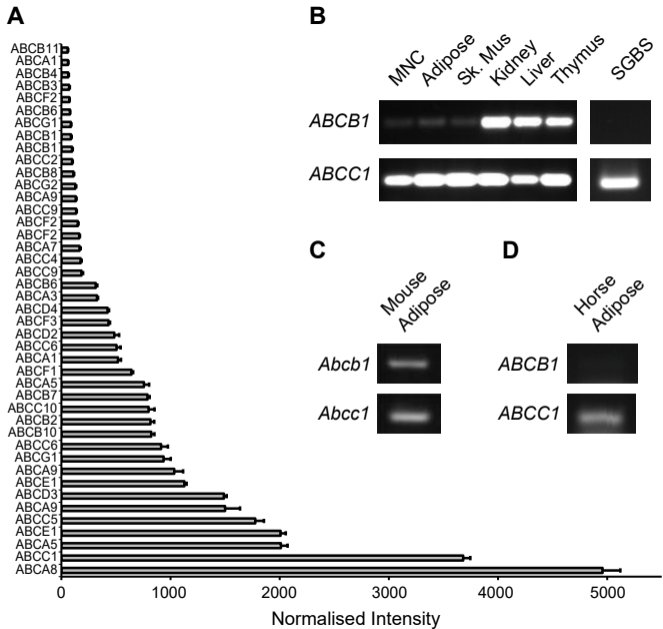
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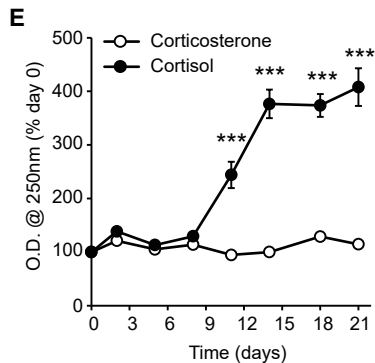
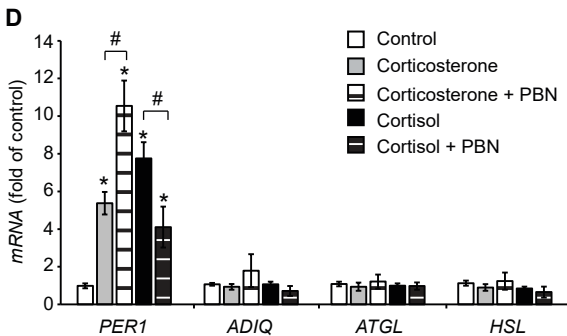
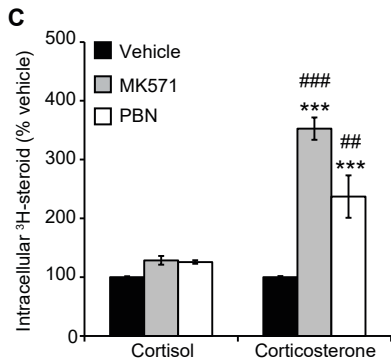
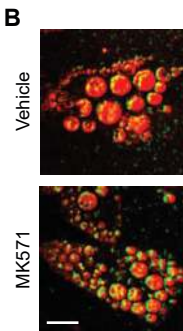
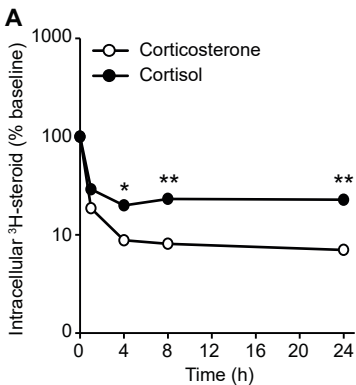
795 **Fig. 7.** Differential effects of cortisol and corticosterone in brain versus adipose are conferred by  
796 tissue-specific expression of ABC transporters. In conventional glucocorticoid replacement  
797 therapy, cortisol (hydrocortisone) action in the brain is limited by export through ABCB1, but  
798 the absence of ABCB1 in human adipose tissue allows potent effects of cortisol on peripheral  
799 metabolism. With corticosterone therapy, the lack of transport by ABCB1 in the brain allows  
800 corticosterone to exert a potent effect to suppress ACTH, but in adipose tissue, corticosterone  
801 action is limited by ABCC1, protecting against adverse effects of corticosterone on peripheral  
802 metabolism.

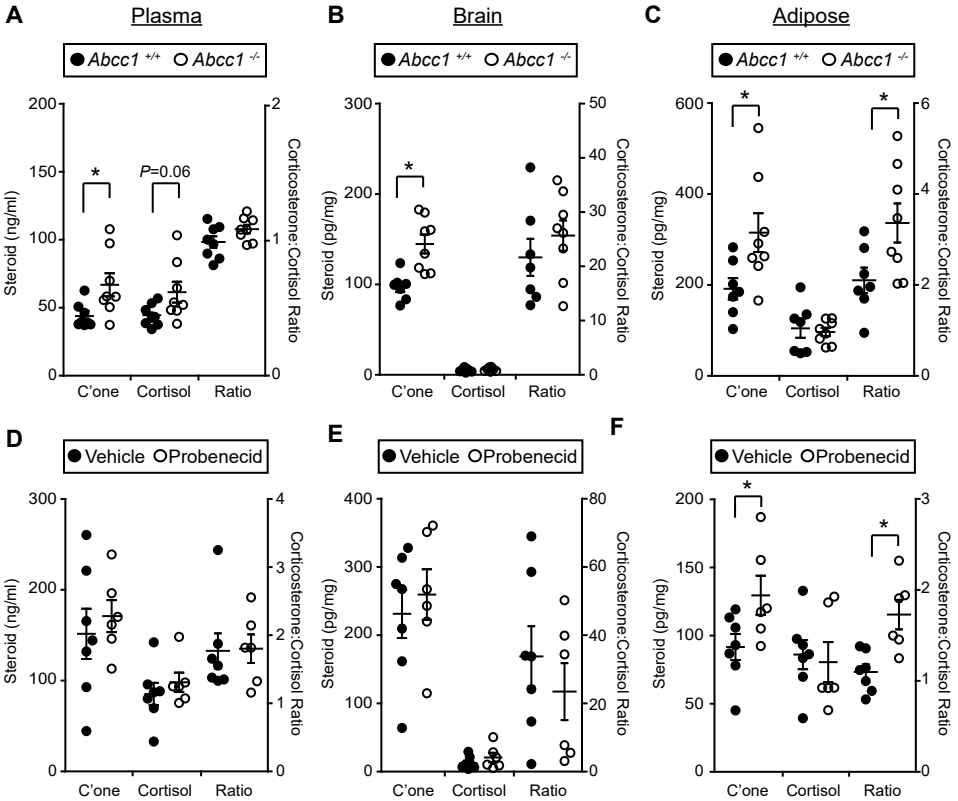
803 **Table 1.** Plasma biochemistry during infusion of either deuterated cortisol or corticosterone in patients with Addison's disease. Data  
 804 are mean  $\pm$  SEM of within-subject averages from samples obtained during each steady state infusion period.  $N = 9$ . Comparisons were  
 805 by two-way repeated measures ANOVA: only ACTH ( $P < 0.001$ ), non-esterified fatty acids (NEFA, ( $P = 0.018$ ), and insulin ( $P = 0.042$ )  
 806 changed with duration of steroid infusion, but there were no differences between corticosterone or cortisol infusions and no significant  
 807 interactions between steroid and duration.

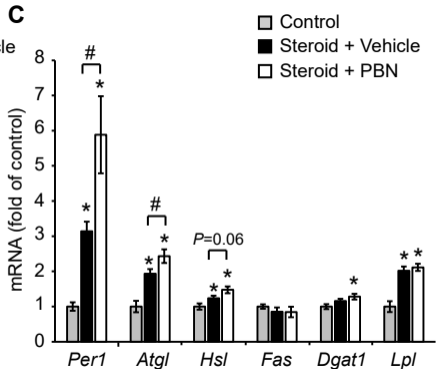
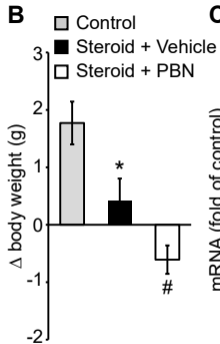
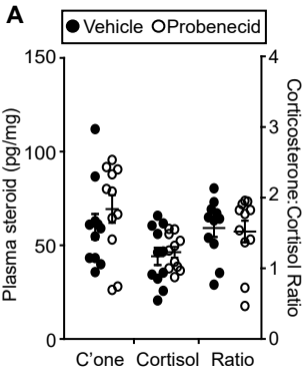
	Cortisol				Corticosterone			
Duration of infusion (min)	0-60	80-140	160-240	260-330	0-60	80-140	160-240	260-330
Total deuterated steroid (nM)	0	53 $\pm$ 11	190 $\pm$ 37	419 $\pm$ 74	0	34 $\pm$ 4	156 $\pm$ 15	340 $\pm$ 26
Endogenous cortisol (nM)	18 $\pm$ 4	12 $\pm$ 2	8 $\pm$ 2	5 $\pm$ 1	22 $\pm$ 7	16 $\pm$ 5	11 $\pm$ 3	7 $\pm$ 2
ACTH (pM)	42 $\pm$ 5	40 $\pm$ 5	37 $\pm$ 5	30 $\pm$ 5	42 $\pm$ 4	43 $\pm$ 4	38 $\pm$ 5	33 $\pm$ 5
Glucose (mM)	4.5 $\pm$ 0.1	4.3 $\pm$ 0.1	4.4 $\pm$ 0.2	4.6 $\pm$ 0.2	4.7 $\pm$ 0.2	4.8 $\pm$ 0.2	4.7 $\pm$ 0.2	4.8 $\pm$ 0.2
Insulin (pM)	44 $\pm$ 11	39 $\pm$ 7	38 $\pm$ 7	35 $\pm$ 6	43 $\pm$ 8	39 $\pm$ 6	37 $\pm$ 6	33 $\pm$ 5
NEFA ( $\mu$ M)	333 $\pm$ 54	415 $\pm$ 59	474 $\pm$ 63	477 $\pm$ 60	338 $\pm$ 47	391 $\pm$ 50	420 $\pm$ 66	412 $\pm$ 58

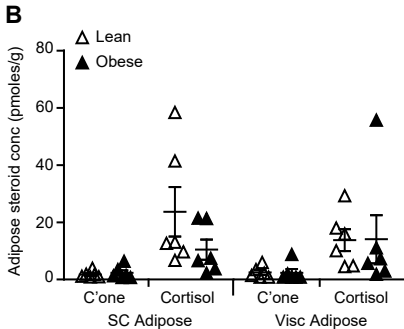
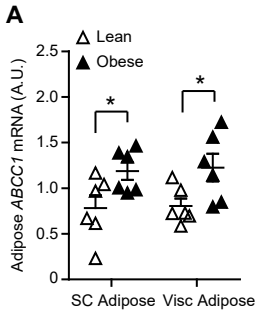
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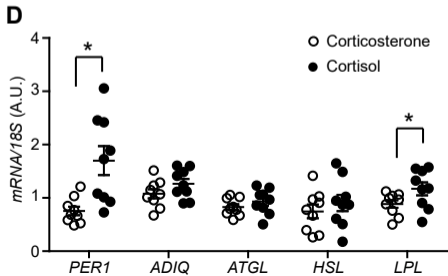
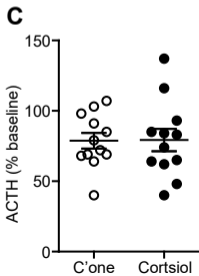
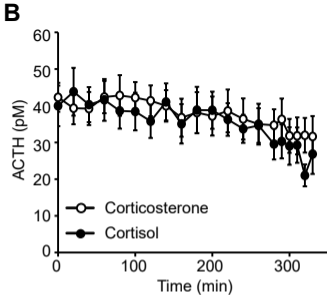
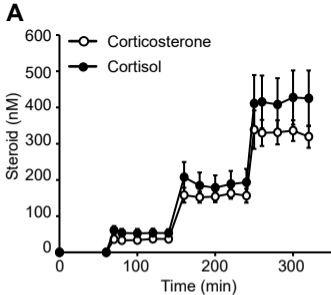










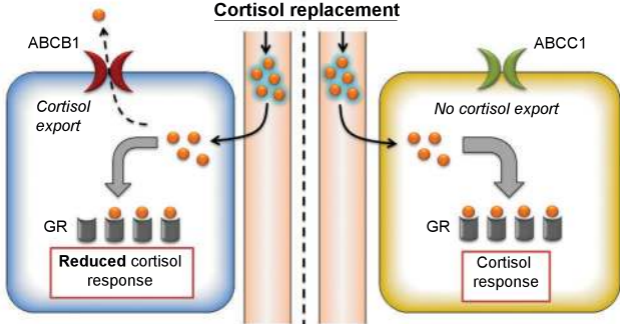




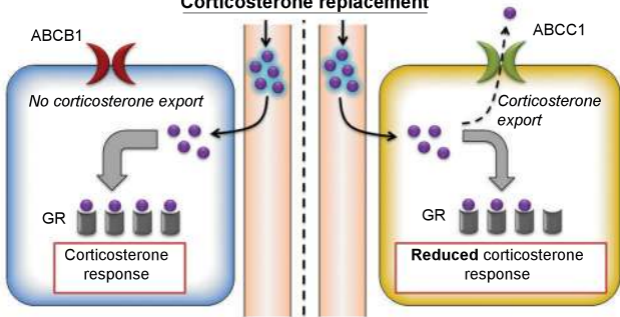
Brain

Adipose

**Cortisol replacement**

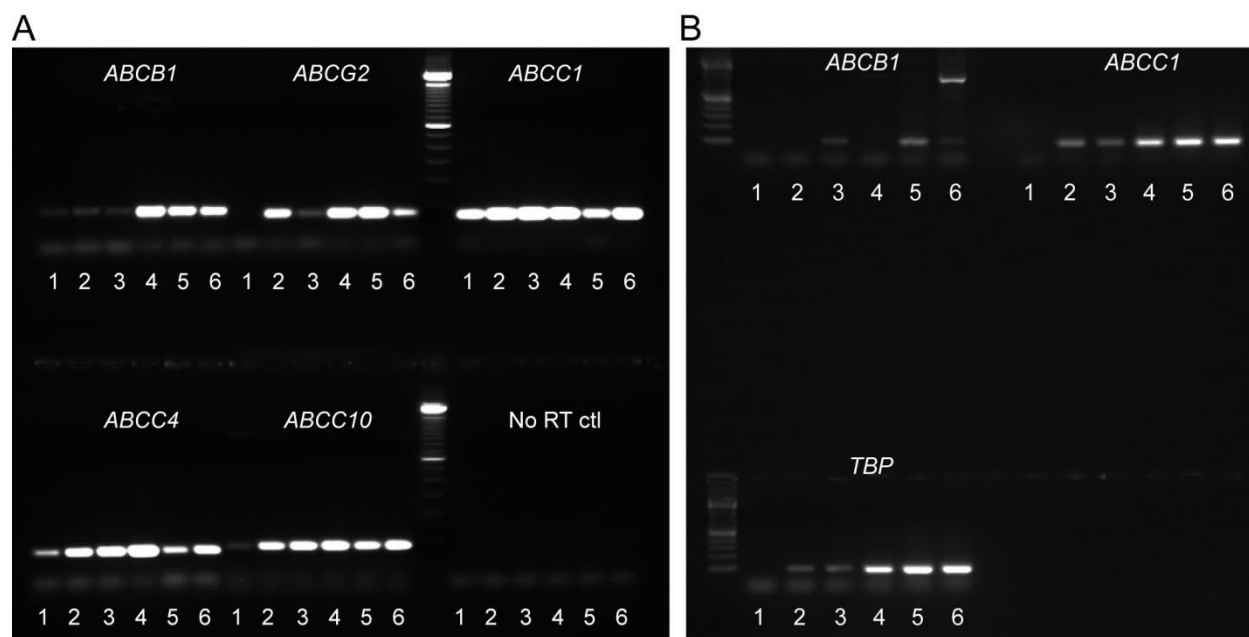


**Corticosterone replacement**



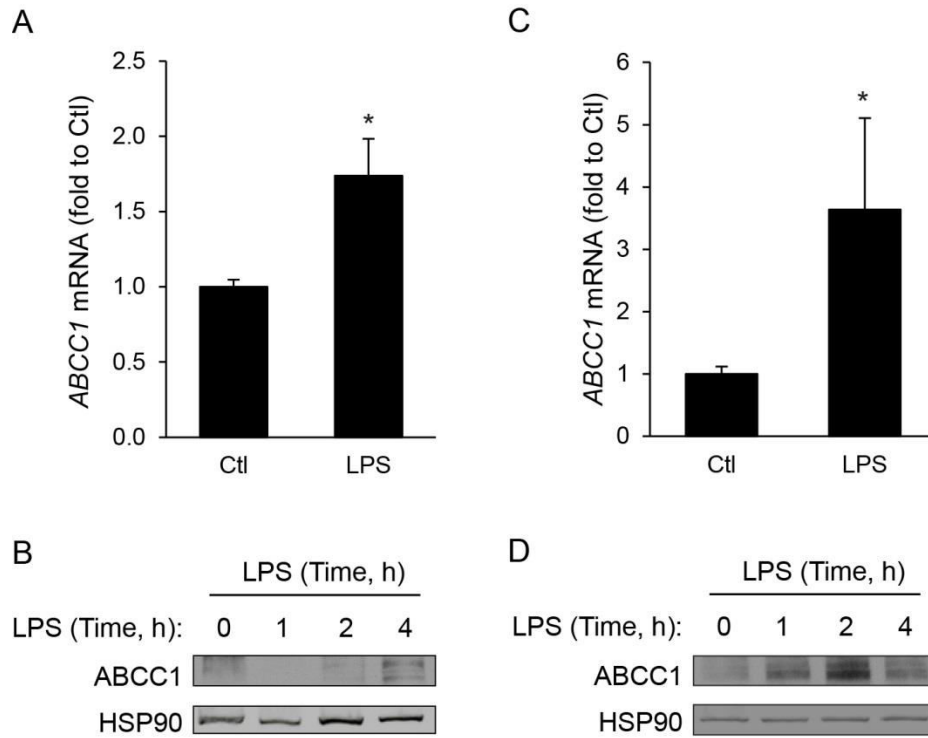
**Key:** ● Cortisol ● Corticosterone (X) ABCB1 (X) ABCC1 GR

1 **Supplementary Materials:**



2  
3 **Fig. S1.** Whole gel PCR image of ABC transporter expression in tissues and cells. (A) PCR gel  
4 of ABC transporter expression from human tissues. Lanes are denoted as follows: 1 =  
5 mononuclear cells, 2 = adipose, 3 = skeletal muscle, 4 = kidney, 5 = liver, 6 = thymus. Genes are  
6 indicated above bands. No RT ctl = negative control (without reverse transcriptase enzyme) for  
7 each sample. (B) Whole gel PCR image of *ABCB1*, *ABCC1*, and *TBP* in various cell lines. Lanes  
8 are denoted as follows: 1 = negative control, 2 = A549, 3 = HepG2, 4 = SGBS, 5 = HeLa, 6 =  
9 MCF-7.

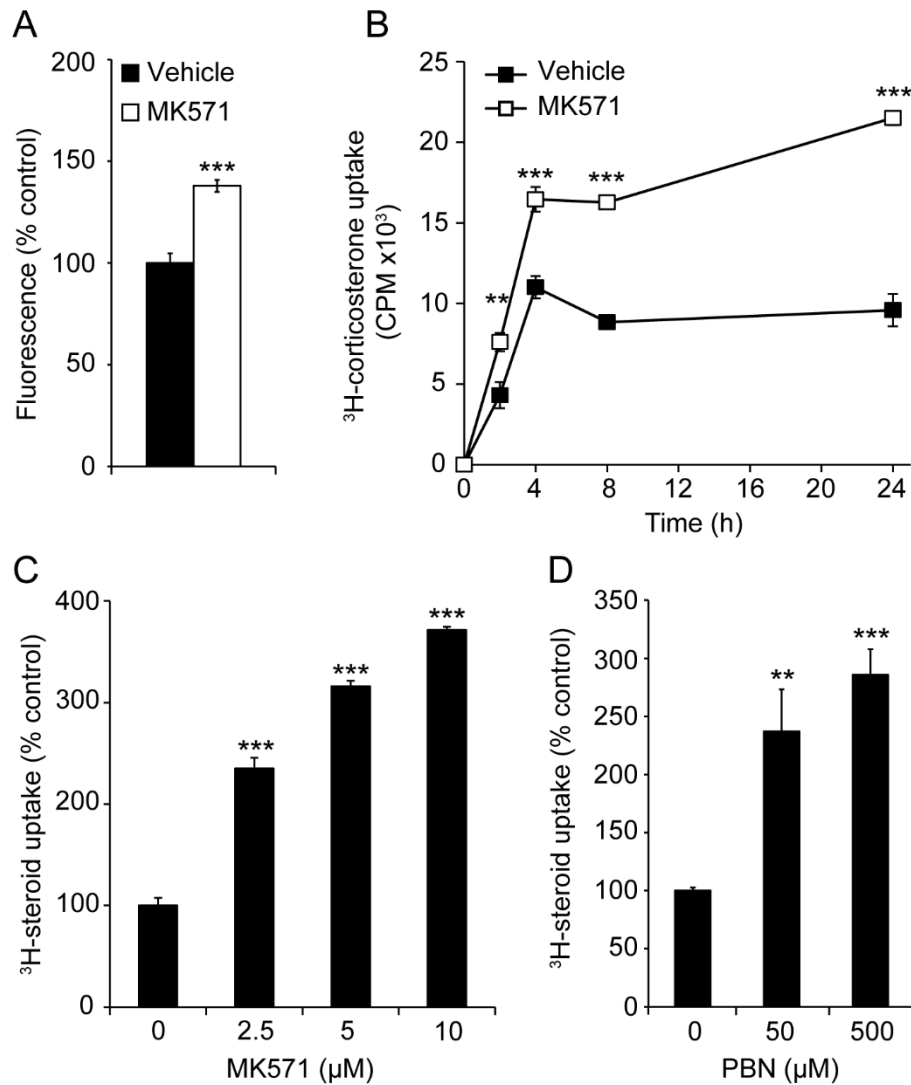
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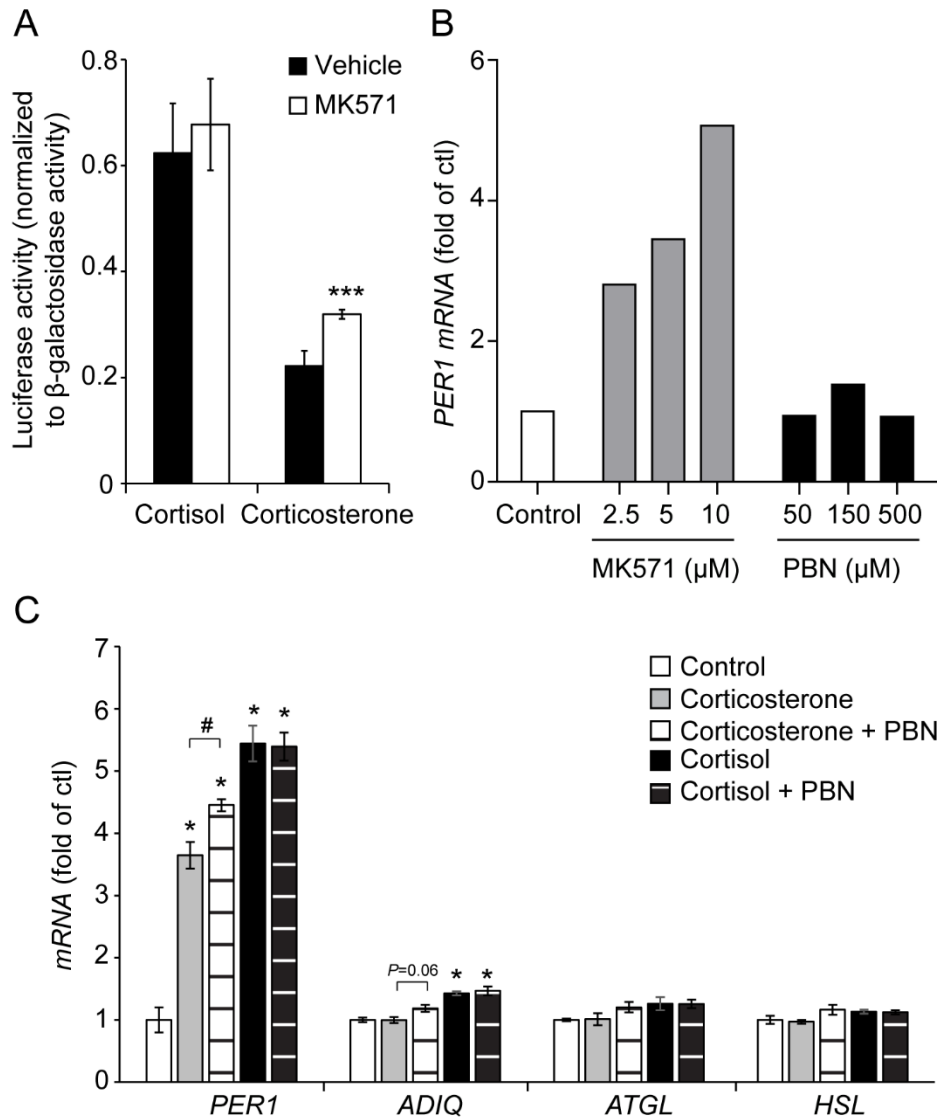
12

13 **Fig. S2.** Correlation of *ABCC1* mRNA and protein concentrations in human adipocytes. (**A** and  
 14 **B**) SGBS adipocytes and (**C** and **D**) primary human adipocytes treated with lipopolysaccharide  
 15 (100 ng/ml) for the indicated times. mRNA concentrations after 2 hours of treatment (**A** and **C**)  
 16 correlate with protein concentrations (**B** and **D**). Data for mRNA are mean  $\pm$  SEM,  $n = 3$ , \*  
 17  $P < 0.05$  vs control (Ctl).



18  
 19 **Fig. S3.** ABCC1 inhibition in SGBS adipocytes. (A) SGBS adipocytes incubated with Calcein-  
 20 AM for 90 min demonstrate the extent of ABCC1 inhibition by MK571 (10  $\mu$ M;  $n = 6$ , Student's  
 21 t-test: \*\*\* $P < 0.001$  vs control). (B) Time course of  $^3\text{H}$ -corticosterone accumulation with ABCC1  
 22 inhibition (MK571, 10  $\mu$ M;  $n = 3$ , two-way ANOVA with Bonferroni post-hoc test: \*\* $P < 0.01$ ,  
 23 \*\*\* $P < 0.001$ ). Dose response of  $^3\text{H}$ -corticosterone accumulation over 24 hours after ABCC1

- 24 inhibition with various concentrations of **(C)** MK571, or **(D)** probenecid (PBN;  $n = 3$ , one-way
- 25 ANOVA: \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs 0). All data are mean  $\pm$  SEM.

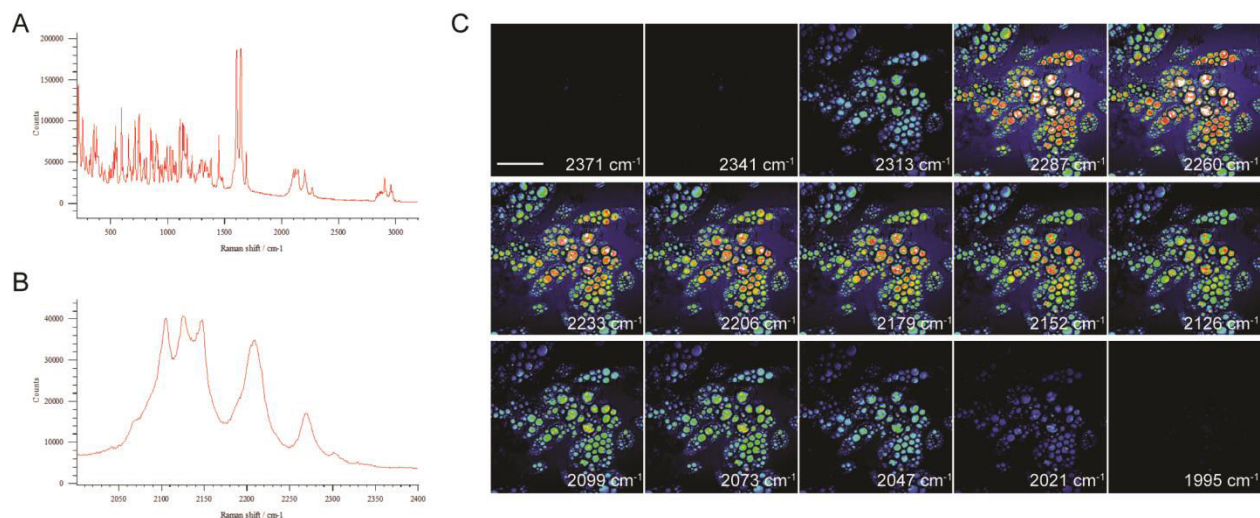


26

27 **Fig. S4.** Effects of ABCC1 inhibition on GR-mediated transcription. (A) A549 cells transfected  
 28 with glucocorticoid-responsive MMTV-luciferase reporter and treated with corticosterone or  
 29 cortisol (500 nM, 24 hours) show potentiation of response to corticosterone but not cortisol by  
 30 ABCC1 inhibition (MK571, 10  $\mu$ M;  $n = 3$ , two-way ANOVA with Bonferroni post-hoc test:  
 31 \*\*\* $P < 0.001$  vs vehicle). (B) Glucocorticoid-responsive period circadian clock 1 (*PER1*) gene  
 32 expression in SGBS adipocytes treated with MK571 or probenecid (PBN) for 24 hours,  $n = 2$ .

33 (C) Glucocorticoid-responsive gene expression in SGBS adipocytes treated with corticosterone  
34 or cortisol (500 nM) in the presence of absence of probenecid (PBN, 50  $\mu$ M) for 24 hours.  
35 Glucocorticoid-responsive genes are *PER1*, adiponectin (*ADIQ*), adipose triglyceride lipase  
36 (*ATGL*), and hormone sensitive lipase (*HSL*), ( $n = 3$ , Student's t-test: \* $P < 0.05$  vs control;  
37 # $P < 0.05$  vs PBN). All data are mean  $\pm$  SEM.

38



39  
40 **Fig. S5.** Optimization of CARS microscopy for detection of intracellular D8-corticosterone. (A)  
41 Raman spectra of D8-corticosterone showing peaks representing C-D bonds between 2000 and  
42 2300  $\text{cm}^{-1}$ . (B) Magnified Raman spectrum of D8-corticosterone between 2000 and 2400  $\text{cm}^{-1}$ .  
43 (C) Coherent Anti-stokes Raman Scattering (CARS) images of SGBS adipocytes treated with  
44 D8-corticosterone (10  $\mu\text{M}$ , 24 hours) probed at inverse wavenumbers corresponding to the  
45 Raman spectrum and showing optimal detection at 2126  $\text{cm}^{-1}$  (scale bar = 100  $\mu\text{m}$ ).



46 **Table S1.** Characteristics of lean and obese study participants providing adipose biopsy samples  
 47 during surgery.

48

	Lean	Obese
Age (years)	60 ± 4	59 ± 3
Gender (M/F)	2/4	2/4
Body mass index (kg/m <sup>2</sup> )	23.9 ± 0.3	41.4 ± 1.9

49

50 **Table S2.** Characteristics of Addison’s study participants.

51

	Mean ± SEM	Range
Age (years)	53 ± 5	20 - 65
Gender (M/F)	3/6	
Body mass index (kg/m <sup>2</sup> )	24.7 ± 1.2	21.7 - 32.9
Years since diagnosis	19 ± 3	4 - 30
Daily hydrocortisone dose (mg)	21 ± 2	15 - 30
Daily fludrocortisone dose (µg)	76 ± 30	0 - 300

52

53

54 **Table S3.** Summary of studies describing plasma corticosterone and cortisol concentrations in healthy subjects.

Reference	Gender (M/F)	Age (years)	Corticosterone (B) nM	Cortisol (F) nM	B/F <sup>‡</sup>	n	Time	B assay Technique
Sweat, 1955 (23)	ns	ns	124.1 ± 14.4	298.0 ± 16.6	0.416	21	ns	Fluorometric method
Peterson, 1957 (24)	18/12	ns	31.7 ± 11.5	386.2 ± 33.1	0.082	30	ns	Isotope dilution/fluorescence
Ely et al, 1958 (25)	ns	ns	86.6 ± 5.8	300.7 ± 15.1	0.288	20	ns	Fluorometric method
Fraser et al, 1968 (26)	ns	ns	19.0 (3.8-66.4)	270.3 (85.5-557)	0.067	29	ns	Double isotope assay
Huther et al, 1970 (27)	M F	26.8 ± 2.6 31.3 ± 4.4	47.2 ± 6.0 49.7 ± 6.5	403.0 ± 37.5 422.4 ± 67.8	0.124 0.118	9 <sup>†</sup> 10 <sup>†</sup>	1045-1145*	Fluorometric method
Dluhy et al, 1972 (28)	8/2	21-34	26.3 ± 3.2	634.5 ± 55.2	0.041	10	0900	Radioimmunoassay
Newsome, Jr. et al 1972 (29)	ns	ns	11.5 ± 0.9	339.3 ± 22.1	0.034	8	ns	Competitive protein binding
Oddie et al, 1972 (30)	ns	ns	12.1 ± 2.6	383.4 ± 4.0	0.030	18	0900	Double isotope assay
West et al, 1973 (31)	M	19-50	11.4 ± 1.7	386 ± 36	0.030	15	0800	Radioimmunoassay
	F <sup>1</sup>		20.4 ± 2.0	386 ± 36	0.053	9		
	F <sup>2</sup>		16.7 ± 2.1	359 ± 21	0.047	9		
Nishida et al, 1977 (32)	ns	ns	23.0 ± 3.0	419.6 ± 28.7	0.055	10	0900	Radioimmunoassay
Karssen et al, 2001 (10)	M	57 ± 1.9	16.4 ± 3.1	350.0 ± 81.5	0.047	11	ns	LC-MS
Raubenheimer et al, 2006 (13)	M	39.2 (23-70)	58.4 ± 9.2	830.4 ± 68.4	0.069	16	0830-0900	Radioimmunoassay

55  
56 Mean ± SEM or range (). ns = not specified  
57 † = mean of 5 samples for each subject  
58 \* = approximate time inferred from clinical protocol  
59 <sup>1</sup> follicular phase, <sup>2</sup> luteal phase  
60 ‡ mean [B] (nM)/mean [F] (nM)

61 **Table S4.** Primer sequences for PCR and corresponding expected product size.

Gene ID	Primer sequence 5' to 3'	Expected product size (bp)
<i>ABCC1</i> Human	F: gcctattaccccagcatcg	69
	R: gatgcagttgccacaca	
<i>ABCB1</i> Human	F: aagcatttacttcaaacttgta	78
	R: tggattcatcagctgcathtt	
<i>ABCG2</i> Human	F: tggcttagactcaagcacagc	67
	R: tcgtcctgcttagacatcc	
<i>ABCC4</i> Human	F: cctggcgaattgtagctgt	68
	R: agcacggcacttaacagtga	
<i>ABCC10</i> Human	F: agctcactgccaccaagg	76
	R: caaggaagttgttgagagga	
<i>Abcc1</i> Mouse	F: ggaatttcggctgagtgc	63
	R: agccaatattgctgcacct	
<i>Abcb1</i> Mouse	F: tgctttgtgggcaaaggtgta	106
	R: cacagttctgatggctgctaa	
<i>ABCB1</i> Horse	F: tcaggtggcctggataa	157
	R: cgaactgtagacaagcgatga	
<i>ABCC1</i> Horse	F: caaaatcatggctgcctaaa	89
	R: gaaagtgacatcgcaaacaa	

63 **Table S5.** Human primer sequences for qPCR and corresponding probe number from Roche  
 64 Universal Probe Library (UPL).

Gene ID	Primer sequence 5' to 3'	Roche UPL Probe Number
<i>ABCC1</i> Human	F: gcctattaccccagcatcg	28
	R: gatgcagttgccacaca	
<i>ABCB1</i> Human	F: aaggcatttacttcaaactgtca	18
	R: tggattcatcagctgcatTTT	
<i>PER1</i> Human	F: ctcttccacagctccctca	87
	R: ctttggatcggcagtggt	
<i>LPL</i> Human	F: atgtggcccggtttatca	25
	R: ctgtatccaagagatggacatt	
<i>ADIPONECTIN</i> Human	F: ggtgagaagggtgagaaagga	85
	R: ttcaccgatgtctcccttag	
<i>ATGL</i> Human	F: ctccaccaacatccacgag	89
	R: ccctgcttgcacatctctc	
<i>18S</i> Human	F: ctccacaggaggcctacac	46
	R: cgcaaaatatgctggaacttt	

65 **Table S6.** Murine primer sequences for qPCR and corresponding probe number from Roche  
66 Universal Probe Library (UPL).

Gene ID	Primer sequence 5' to 3'	Roche UPL Probe Number
<i>Abcc1</i> Murine	F: ggaatttcggtgagtgc	105
	R: agccaaatattgctcacct	
<i>Abcb1</i> Murine	F: tgctttgtgggcaaaggta	78
	R: cacagttctgatggctgctaa	
<i>Per1</i> Murine	F: gcttcgtggactgacacct	71
	R: tgcttagatcggcagtggt	
<i>Lpl</i> Murine	F: ctgctctcagatgcctac	95
	R: ggttggttgcttgcatt	
<i>Atgl</i> Murine	F: gagcttcgctcaccaac	89
	R: cacatctctcggaggacca	
<i>Hsl</i> Murine	F: gcgctggaggagtgtttt	3
	R: ccgctctccagttgaacc	
<i>Fas</i> Murine	F: ccaaatccaacatgggaca	34
	R: tgctccaggataacagca	
<i>Dgat1</i> Murine	F: gcttctgcagtttgagacc	31
	R: tccagttctgcaaaagtaggt	
<i>Tbp</i> Murine	F: gggagaatcatggaccagaa	97
	R: gatgggaattccaggagtca	
<i>18S</i> Murine	F: ctcaacacgggaaacctcac	77
	R: cgctccaccaactaagaacg	

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